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A MULTI-OMICS APPROACH TO UNCOVER ESTROGEN RECEPTOR (ER) AND ACTIVATOR PROTEIN 1 (AP-1) SIGNALING NETWORKS IN BREAST CANCER

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A multi-omics approach to uncover estrogen receptor (ER) and activator protein 1 (AP-1) signaling networks in breast cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my dearest family

ABSTRACT

Estrogen receptor (ER) binds to DNA indirectly through other transcription factors (e.g. AP-1) to modulate gene expression, which is a tethering mechanism. The ER/AP-1 crosstalk plays an important role in tamoxifen therapy resistance. However, the overlap in DNA binding profiles of ER and AP-1 transcription factors at genome-wide level has not been described. Moreover, AP-1 plays a pivotal role in various cellular processes in breast cancer. The transcriptional activity of AP-1 is controlled by coregulators, thereby regulating the expression of specific genes. Understanding protein-protein interactions is fundamental to the mechanism of AP-1 signaling. In addition, ERα is one of the key biomarkers for diagnosis and endocrine therapy of breast cancer. However, ERα status is not considered to be a perfect marker for responsiveness to anti-estrogens. It has been shown that ERβ may act as a tumor suppressor and could be a therapeutic target for breast cancer, however the functions of ERβ in this setting remain to be further explored. The use of multi-functional genomic technologies to identify cistrome, transcriptome and proteome of ER or AP-1 has resulted in comprehensive deciphering of the role of the ER and AP-1 in breast cancer, which also provides information for developing novel therapeutic strategies for breast cancer.

In Paper I, we investigated the genome-wide assessment of c-Jun, a potent member of AP-1 family, and ERα cistrome and transcriptome in ERα-positive breast cancer cells. Our findings demonstrate the genome-wide co-localization of ERα and c-Jun binding regions and suggest that ERα tethering to AP-1 is a global mechanism for gene transcription regulated by ERα. In addition, the results confirm that the sensitivity of ERα-positive breast cancer cells to tamoxifen therapy is reduced by c-Jun overexpression. Moreover, it is shown that expression of transforming growth factor β induced (TGFBI) protein is associated with poor outcomes of ERα-positive breast cancer patients receiving endocrine therapy and thus as a candidate gene that may cause tamoxifen resistance through ERα and AP-1 crosstalk.

In Paper II, we elucidated the first Fra-1 associated interactome in triple-negative breast cancer (TNBC) cells using Rapid Immunoprecipitation Mass Spectrometry of Endogenous proteins (RIME) approach, showing that the most enriched Fra-1 interacting protein was DDX5. The cistrome and transcriptome of DDX5 extensively overlapped with that of Fra-1, which is highly associated with the TNBC cell growth. Furthermore, we found that DDX5 acts as a transcriptional coactivator for Fra-1, enhancing Fra-1-dependent TNBC cell proliferation through increasing the transcriptional activity of Fra-1. We also showed that higher expression level of DDX5 protein was detected in triple-negative basal-like tumors compared with that in non-basal-like ones. In addition, the direct target gene set of DDX5 can predict poor clinical outcome of breast cancer patients.

In Paper III, we generated a novel breast cancer cell model with overexpression of ERβ in the absence of ERα. We used CRISPR/Cas9 system to knock out ERα in MCF7 breast cancer cells with stable Tet-Off-inducible ERβ expression. We found that only ERβ-expressing MCF7 cells displayed a significant reduction in cell proliferation in response to E2 compared

with vehicle, conversely, only ERα-expressing MCF7 cells displayed an increased cell proliferation upon E2 treatment. The RNA-seq results indicated that ERβ could modulate specific gene expression profile different from that of ERα. Furthermore, functional enrichment analysis showed that the two ER isoforms regulate cell proliferation in opposite direction; ERβ is significantly involved in the biological process “negative regulation of cell proliferation”.

In conclusion, the studies presented in the thesis contribute to comprehensive understanding of the mechanism of ER and AP-1 signaling in breast cancer. We characterized two molecules, TGFBI and DDX5, in breast cancer, suggesting that they could be the candidates of therapeutic targets. We also provided evidences that ERα and ERβ have opposite effects on E2-dependent breast cancer cell proliferation by regulating distinct gene sets.

LIST OF SCIENTIFIC PAPERS

I. **He H**, Sinha I, Fan R, Haldosen LA, Yan F, Zhao C, Dahlman-Wright K. *c-Jun/AP-1 overexpression reprograms ERalpha signaling related to tamoxifen response in ERalpha-positive breast cancer.* Oncogene. 2018 May;37(19):2586-2600.

II. **He H**, Song D, Sinha I, Hessling B, Li X, Haldosen LA, Zhao C. *Endogenous interaction profiling identifies DDX5 as an oncogenic coactivator of transcription factor Fra-1.* Oncogene. 2019 Jul:38(28):5725-5738.

III. **He H**, Song D, Sinha I, Haldosen LA, Zhao C. *ERα and ERβ exert differential regulation of gene expression in MCF7 cells.* Manuscript.

Related paper (not included in this thesis)

Qiao Y, He H, Josson P, Sinha I, Zhao C, Dahlman-Wright K. *AP-1 Is a Key Regulator of Proinflammatory Cytokine TNFalpha-mediated Triple-negative Breast Cancer Progression.* J Biol Chem 2016 Mar 4;291(10): 5068-5079.

CONTENTS

LIST OF ABBREVIATIONS

AF	Activation function
AIs	Aromatase inhibitors
AP-1	Activating protein 1
AR	Androgen receptor
ATF	Activating transcription factor
BL	Basal-like
bZIP	Basic leucine zipper
CAF	Cancer-associated fibroblast
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	cAMP-responsive-element-binding proteins-binding proteins
ChIP	Chromatin immunoprecipitation
CDK	Cyclin-dependent kinases
CRE	cAMP response elements
CREB	cAMP-responsive-element-binding proteins
CRISPR	Clustered regularly interspaced short palindromic repeats
CRTC1	CREB-related transcription coactivitor 1
DBD	DNA binding domain
DCIS	Ductal carcinoma in situ
Dox	Doxycycline
E2	17β-estradiol
ECM	Secreted extracellular matrix
EGF	Epidermal growth factor
EGFP	Green fluorescent protein
EMT	Epithelial-mesenchymal transition
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERE	Estrogen response element
ER	Estrogen receptor
ERKO	ER knock-out
FACS	Fluorescence-activated cell sorting
FAS1	Fasciclin-1

FosL	Fos related antigen
GEP	Gene expression profiling
GF	Growth factor
GFR	Growth factor receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HDR	Homology-directed repair
HER2	Human epidermal growth factor receptor 2
HRG	Heregulin
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
JDP	Jun-dimerizing partner
JNK	c-Jun N-terminal kinase
LAR	Luminal androgen receptor
LBD	Ligand-binding domain
lncRNA	Long non-coding RNA
M	Mesenchymal
MAF	Musculoaponeurotic fibrosarcoma
MARE	MAF-recognition element
mTOR	Mammalian target of rapamycin
N-CoR	Nuclear corepressor
NF-κB	Nuclear factor-κB
NHEJ	Non-homologous end joining DNA repair
PARP	Poly (ADP-ribose) polymerase
PHB	Peohibitin
qPCR	Quantitative polymerase chain reaction
RIME	Rapid immunoprecipitation mass spectrometry of endogenous proteins
RIP140	Receptor-interacting 140
rtTA	Reverse tetracycline-controlled transactivator

SERD	Selective ER down-regulator
SERM	Selective ER modulator
SP	Secretory signal peptide
Sp-1	Stimulating protein-1
SRC	Steroid receptor coactivator
Tet	Tetracycline
tetO	Tet operator sequences
TetR	Tet repressor protein
TF	Transcription factor
TGFβ	Transforming growth factor β
TGFBI	Transforming growth factor β induced protein
TNBC	Triple negative breast cancer
TNFα	Tumor necrosis factor α
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRE	TPA-response elements
tTA	Tetracycline-controlled transactivator
αERKO	ERα knock-out
βERKO	ERβ knock-out
βig-h3	TGF-β-induced gene-human, clone 3

1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is a malignant tumor arising from the cells in breast, and is the most frequent cancer among women around the world [1, 2]. GLOBOCAN 2018 database reports that the estimated number of new cases for breast cancer was more than 2 million among women, accounting for 24.2% of all new cancer cases in women [3]. Various risk factors and the usage of mammography can influence the patterns of global incidence [4]. The highest female breast cancer incidence rates (age-standardized) are in Australia and New Zealand, Western and Northern Europe and North America, while lowest rates are in most regions of Africa and Asia [5]. With the development of treatment and diagnostic techniques for breast cancer, the mortality rates have decreased. For instance, in the United State, breast cancer death rates decreased by 39% from 1989 to 2015, and the five-year survival rate has increased from 63% in 1960 to 90% in 2018 [6, 7]. However, breast cancer remains the major cause of death for women, with 15% of breast cancer-related deaths around world [4]. Especially in the developing low-income and middle-income countries, breast cancer mortality is high [4, 8, 9].

1.1.1 Risk factors for breast cancer

Along with the increased age, the incidence and mortality rates of breast cancer increase proportionally. Globally, the patients of breast cancer have a sharp incline beginning at age 40 and reach a peak at around age 60 [10]. Currently, among the causes of cancer-related deaths, breast cancer becomes the leading one for young woman (under 45 years old). In USA, about 10,000 women with age less than 40 are diagnosed with invasive breast cancer [11]. In Asia, 13% of women diagnosed with breast cancer are less than 40 years of age, while 5% are less than 35 years [12]. As breast cancer has a genetic component, family history is another important risk factor. The risk of women to develop breast cancer increases with the number of affected relatives, particularly with the first-degree relatives [13, 14]. The hereditary breast cancer characterized with the mutations in high-penetrance genes, such as *BRCA1* and *BRCA2*, constitutes 3-6% of all breast cancers [15]. Reproductive factors (early menarche (<12 years old), late age at first full-term pregnancy (>30 years old), nulliparity and late menopause (>55 years old)), menopausal hormone therapy and breast characteristics (personal history of breast cancer (<40 years), ductal or lobular carcinoma in situ and increased mammographic breast density) increase the risk for breast cancer [11, 16]. Moreover, some lifestyle and environmental factors that are risk for breast cancer to develop are alcohol consumption, lack of physical activity, obesity (postmenopausal) and radiation exposure to chest, etc. [10, 17].

1.1.2 Molecular classification of breast cancer

The purposes of classifications of breast cancer, according to different criteria, are to diagnose and management the disease accurately. Breast cancer is traditionally classified by

the clinicopathologic features, including histopathological type, tumor grade and tumor stage (TNM), and biomarker receptor status, together with the characteristics of patients (e.g. age and menopausal status) [18, 19].

The advent of high-throughput technologies for gene expression analysis has considerably influenced our understanding of breast cancer biology. Global gene expression profiling (GEP) studies are used to classify breast cancer into four intrinsic subtypes by hierarchical clustering analysis [20-22], including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2, also called ERBB2)-enriched and basal-like tumor. Subsequently, immunohistochemistry (IHC)-based surrogate molecular classification is applied in the daily clinical practice, because the IHC surrogate biomarkers (ER, PR, HER2 and Ki-67) are correlated well with these intrinsic subtypes and IHC approach is a low cost and widely used technique [23, 24]. Comparing with GEP-based classification, IHC-based classification can divide breast cancer patients into similar subgroups of clinical outcomes [24]. However, some discrepancies exist between these two classifications. For instance, basal-like subtype is often identified as triple negative breast cancer (TNBC) in clinical practice because a majority of basal-like subtype tumors are typically negative for ER, PR and HER2 defined by IHC. These two terms share considerable and significant overlap but not complete. Several studies reported that around 70% of IHC-based TNBCs were GFP-based intrinsic basal-like subtype, while 18-40% of basal-like breast cancers were not triple-negative immunophenotype [25-27]. The details about intrinsic subtypes are shown in Table 1.

Table 1. Breast cancer molecular subtypes

Intrinsic subtypes	Gene expression profile	IHC-based	Treatment
Luminal A	High expression of luminal epithelial genes and ER-related genes	ER+, PR≥20%, HER2-, Ki-67 low	Endocrine therapy
Luminal B	Lower expression of luminal epithelial and ER-related genes, whereas higher level of proliferation and HER2-related genes than luminal A	ER+, PR<20%/ or HER2+/ or Ki-67 high	Endocrine therapy + Chemotherapy
HER2-riched	High expression of HER2-related genes, low expression of ER and related genes	ER-, PR-, HER2+	Chemotherapy+ Anti-HER2
Basal-like	High expression of basal epithelial and proliferation genes, low expression of ER- and HER2-related genes	Triple negative: ER-, PR-, HER2-	Chemotherapy

In total, breast cancer is a heterogeneous disease with different histological and biological properties. Molecular classification provides convincing proof supporting the relevance of histopathologic features in the underlying tumor biology. Therefore, histopathologic classification should be used together with molecular classification. Furthermore, gene expression profiling can provide more detailed biological characterization of genomic alterations associated with precise prognostication and risk stratification, which is useful for identifying novel targeted treatment for individual breast cancer patient.

1.1.3 ER-positive breast cancer and endocrine therapy

The seminal class-discovery studies indicated that ER-negative and ER-positive breast cancers, in molecular terms, are primarily distinct diseases [20, 21]. ER-positive breast cancers fall into the luminal A and B subtypes, accounting for about 70% of breast cancer. Luminal A accounts for 50-60% of invasive breast cancers and is the most common molecular subtype. Luminal A breast cancers are of low histological grade and have the best prognosis among all intrinsic subtypes [28, 29]. The breast cancers of luminal B subtype make up 10-20% of all breast cancers. The main biological distinction between the luminal A and B is the expression level of proliferation genes, for example, luminal B has higher expression levels of MKI67 and cyclin B1 and also often expresses EGFR and HER2 [30].

Clinically, luminal A breast cancers are more likely to respond well to endocrine therapy alone [31]. On the other hand, luminal B tumors have increased proliferation and they are more likely to recur with endocrine therapy alone, while they are likely to be more chemo-sensitive. Thus, chemotherapy is recommended in addition to endocrine therapy for luminal B breast cancer patients [32]. Meta-analyses have demonstrated the benefits of adjuvant chemotherapy in reducing recurrence and breast cancer mortality, especially, with a greater magnitude of benefit in those with ER-negative disease, but the absolute benefits may be small and not worth the added risk of toxicity among women who have a baseline low risk of recurrence [33].

Endocrine treatment is a pivotal treatment for women with ER-positive tumors. The majority of endocrine therapies are to inhibit breast tumor growth through depriving the cell of estrogen or blocking its receptor [34]. Anti-estrogens (such as Tamoxifen and Fulvestrant) and aromatase inhibitors (AIs) are currently used to treat ER-positive breast cancer.

Tamoxifen, as one of the selective ER modulators (SERMs), acts as antagonist binding to the ER to block estrogen from attaching to the receptor. Currently, tamoxifen is still one of the frontline and most successful drugs used in pre- and postmenopausal women. Furthermore, it displays a significant long-term benefit for treated patients [35]. For instance, a meta-analysis of 20 trails reported that adjuvant tamoxifen treatment reduces the risk of recurrence by nearly 50% during year 0 to 4 and more than 30% during year 5 to 9 [36]. Furthermore, the breast cancer mortality was reduced by around 30% throughout the first 15 years [36].

Fulvestrant is a selective ER down-regulator (SERD), which competitively inhibits estradiol binding to ER and once it binds to ER induces degradation of ER by inhibiting receptor dimerization [37]. Compared with tamoxifen, fulvestrant has a higher binding affinity to the ER, 89% of that of estradiol [38], and it acts as a pure anti-estrogen [37]. Fulvestrant has already been introduced as a second-line agent for postmenopausal women with advanced breast cancers [39, 40].

AIs inhibit the endogenous synthesis of estrogen by blocking the activity of aromatase. The third-generation AIs include two classes: steroidal AIs (exemeatane) and non-steroidal AIs (letrozole and anastrozole). AIs are mainly used in postmenopausal women as alternatives to

tamoxifen, and also as options for secondary strategy after tamoxifen [41-43]. For premenopausal patients with ER-positive breast cancer, ovarian function suppression combined with AIs or tamoxifen is more effective than tamoxifen alone [44].

1.1.4 HER2-enriched breast cancer and related adjuvant therapy

HER2-enriched subtype belongs to ER-negative breast cancers based on gene expression profile, since it is characterized by low or absent gene expression of ER and related genes, and overexpression of HER2 and genes located in HER2 amplicon at chromosome 17q12 (e.g. GRB7) [19]. This subtype of breast cancer is highly proliferative, likely to be of high histological grade and P53 mutation and has an aggressive clinical outcome [18]. Not all GFP-based intrinsic HER2-enriched tumors are clinically HER2-positive defined by IHC, and a minority of clinically HER2-positive cancers co-expressing ER is classified as intrinsic luminal B subtype [45, 46].

For HER2-positive breast cancers, the reasonable adjuvant treatment options include chemotherapy and anti-HER2 targeted therapy [34, 47]. For instance, one of the first-line treatments for HER2-positive metastatic breast cancer is trastuzumab, a monoclonal antibody that is directed against HER2 [48]. Furthermore, this therapy has dramatically improved overall survival and disease-free survival in HER2-positive breast cancers [48-50]. However, de novo or acquired resistance is still observed in 66-88% of HER2-positive metastatic breast cancer [51]

1.1.5 TNBC breast cancer and related adjuvant therapy

Although the terms of basal-like and TNBCs have been used interchangeably in past years and basal-like subtype predominates in triple-negative tumors, there is a small group of non-basal-like subtype [19, 52]. Recently, according to comprehensive gene expression profile analysis, TNBCs patients are dived into four distinct subtypes; two were basal-like (BL) but with differences in immune response, one mesenchymal (M), and one luminal androgen receptor (LAR) subtype [53, 54]. Furthermore, the majority of the two BL and M subtypes are basal-like identified by PAM50, while LAR is non-basal-like, enriched in HER2 and luminal subtypes [19, 53, 54].

TNBC is a biologically heterogeneous disease, representing 10%-20% of all invasive breast cancers. According to previous literatures, the main characteristics of TNBCs are similar to that of basal-like cancers; TNBC is significantly more aggressive than other molecular subtypes, for example, TNBCs have more advanced stage and poor survival than non-TNBC subtypes regardless of the stage at diagnosis [55-57]; TNBCs are more likely to have both local and distant recurrence and metastases, such as brain and lungs [58]. Furthermore, women at younger age (<50 years old) and of African American race have been identified as having higher risk for TNBCs [25, 59, 60]. *BRCA1* mutations appear to be a risk factor that causes basal-like breast cancers and a subgroup of triple-negative tumors [52, 61-63].

Currently, the diagnosis of TNBCs has direct clinical implications and is important for tumor management. Cytotoxic chemotherapy remains the major therapeutic choice for TNBC patients, because they lack the expression of the appropriate targets (ER, PR or HER2) for endocrine therapy or anti-HER2 agents [64]. Chemotherapy leads to an initial substantial response rate, whereas it is often followed by poor outcomes, such as frequent relapses and lower overall survival [59, 65, 66]. Altogether, the above urgent necessitates the identification of novel therapeutic strategies for TNBC patients. Currently, poly (ADP-ribose) polymerase (PARP) inhibitors, androgen receptor antagonists, immune checkpoint inhibitors and some others are target therapies in study, which are used as monotherapy or together with other standard or investigational agents [64, 67].

1.1.6 Endocrine resistance

Although the endocrine treatments mentioned above for ER-positive breast cancer patients have led to substantial improvements in outcomes, either intrinsic or acquired resistance limits their benefits. For example, acquired endocrine-resistant disease may represent up to one-quarter of all breast cancers [68-70]. Moreover, the agonist effect of tamoxifen may induce a risk of endometrial cancer after long-term use in postmenopausal women [71]. Generally, AIs and fulvestrant are used to follow or replace when patients are resistant to tamoxifen [71, 72]. However, resistance to AIs and fulvestrant eventually occurs [73-75].

The progression of endocrine resistance is recognized as a gradual and step-wise process. Additionally, due to the complex biology of ER, multiple molecular mechanisms could underlie endocrine resistance (Figure 1). Mutations of ER [76] and crosstalk between ER and other signaling pathways (e.g. EGF/EGFR/HER2 pathway) are considered to be major causes of endocrine resistance [77, 78]. In addition, substitution of ER function by androgen receptor (AR), the upregulation of ER coactivators and alterations in corepressors, downstream signaling pathways and transcription factors (e.g. AKT/PI3K/mTOR, MAPK and NF-kB), overexpression of key cell-cycle regulators, as well as stem cell and immune system could contribute to endocrine resistance [70, 71, 79]. Although several mechanisms have been proposed, the complete and precise explanation behind the phenomenon of endocrine resistance cannot be defined by any of them to date.

Recently, multiple targeted agents are used to overcome endocrine resistance, including the use of inhibitors for cyclin-dependent kinases 4 and 6 (CDK4/6), mammalian target of rapamycin (mTOR), PI3K/AKT and histone deacetylase, as well as investigation of new SERDs/SERMs [70, 72, 80]. In addition, it has been shown that HER2 inhibitors combined with endocrine therapies contain clinical benefit [79].

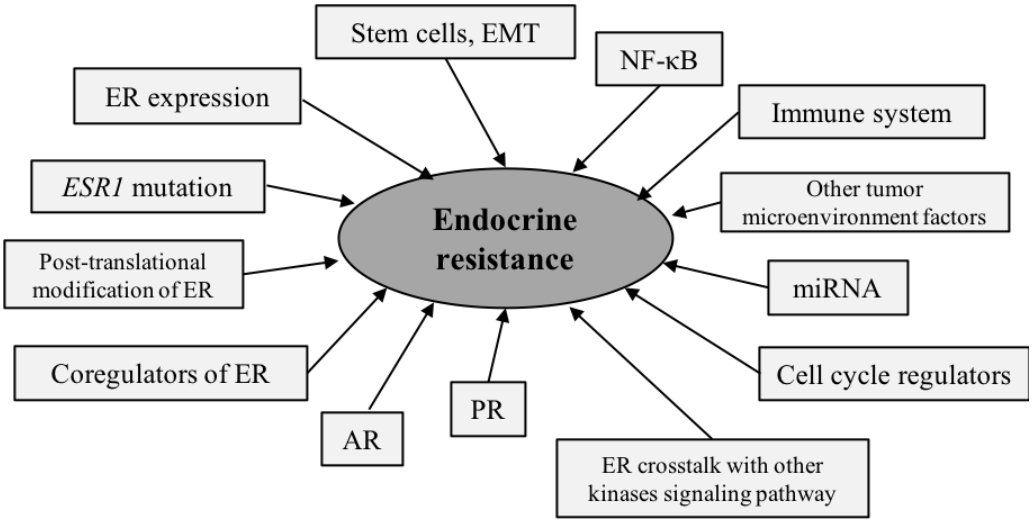

Figure 1. Hypothesized molecular mechanisms of endocrine resistance.

1.2 ESTROGEN RECEPTORS

Estrogen receptors (ERs) belong to the superfamily of nuclear receptors that act as the ligand-inducible transcription factors. ERs include two different members, ERα and ERβ. ERα was the first identified in the 1960s [81] and was cloned in 1986 [82-84]. Subsequently, ERβ was identified and cloned in 1996 from the rat prostate and ovary [85]. Although ERα and ERβ proteins are encoded by separate genes called *ESR1* (on 6q25.1) and *ESR2* (on 14q23), respectively, they have similar overall domain structures.

1.2.1 Structures and functions

ER proteins are comprised of six structural domains named from A to F [86] (Figure 2). A/B domains compose the N-terminal domain, which contains the transcription activation function-1 (AF-1). The role of AF-1 is regulating transcription activation of targeted genes ligand-independently via phosphorylation [87]. The C domain, the DNA binding domain (DBD), can mediate the binding of ERs to specific DNA sequences, such as estrogen response element (ERE). DBD is composed of two zinc-binding motifs and each motif includes an α-helix. The first motif, P-box, determines the DNA-binding specificity, such as the interaction with ERE. The second one contains D-box, which is important for receptor dimerization [88]. The D domain (hinge region) contains a nuclear localization sequence and acts as a flexible connection between DBD and ligand-binding domain (LBD) [89]. The E domain of ERs is the LBD, which consists of 12 helices that hold a dimerization interface, a hormone-binding site and a ligand-dependent coregulator interaction function (activation function 2, AF-2) [89, 90]. F domain following LBD on the far C-terminal and its functions are not fully understood. The F domain of ERα may be able to modulate the activation of transcription, dimerization, interaction of coactivators, and stability of the receptor [88, 91, 92]. ERα and ERβ are highly conserved in the DBD (~95%) and LBD (~60%), but the N-terminal domains and hinge region of two ERs share only ~15% and ~35%, respectively.

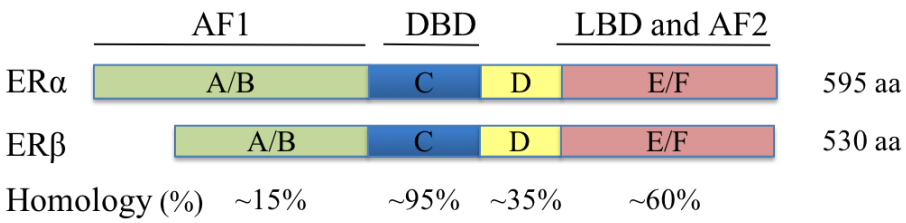

Figure 2. Structure of ERα and ERβ proteins. The letters from A to F represent six functional domains. The numbers on the right side are the total size of protein in amino acids (aa). The numbers (%) on the bottom mean the homology between ERα and ERβ domains.

ERα and ERβ have tissue- and cell-type-specific expression profiles throughout the body. ERα is mainly found in various tissues, including uterus, ovary, breast, kidney, liver, bone and white adipose tissue [93]. ERβ has been reported to express in the ovary, lung, prostate, colon, kidney, male reproductive organs, central nervous system, cardiovascular system, and the immune system [93]. The biological functions are distinct between ERα and ERβ with different expression patterns [94]. Furthermore, ERα and ERβ knockout mice models represent distinct phenotypes [94].

Estrogen is the main natural endogenous ligand for ERs. As the female steroid hormone, estrogen plays an important role in female and male reproduction as well as other systems, such as immune, skeletal, cardiovascular, and central nervous systems [94, 95, 96]. The biological effects of estrogen are mediated by these two ERs [86]. Severe damages of reproductive functions are observed in ER knock-out (ERKO) mice models, for instance, both sexes ERα knock-out (αERKO) mice are infertile [97, 98]. Female ERβ knock-out (βERKO) mice show arrested folliculogenesis and subfertility, while male βERKO mice are fertile [99, 100]. However, the life of mice is possible without either or both ERs [97, 98]. The predominant estrogen in the body is 17β-estradiol (E2), which is an unselective ligand for ERα and ERβ with equal binding affinity.

In addition, estrogen is also associated with many different diseases including a variety of cancers, obesity, metabolic disorder and more [101]. Moreover, estrogen exposure has been found to be strongly associated with increased risk for breast cancer development [102, 103]. Therefore, anti-estrogens or antagonists of ERs that could inhibit ER activity are the main choice for the ER-positive breast cancer treatment. For instance, fulvestrant is a pure antagonist for ERα. Except inactivation of both AF-1 and AF-2, fulvestrant can also impair receptor dimerization after binding with ERα [104]. However, tamoxifen can act on both ERs with agonist or antagonist effects in tissue-specific or cell-type specific manner [105]. Antagonists bind to ERs in a manner similar to estrogen, however, they induce a different conformation of LBD, resulting in recruitment of co-repressors, rather than co-activators, by inhibition of AF-2 [106]. Furthermore, the agonistic activity of tamoxifen, as seen in uterus, appears to be associated with the activation of AF-1 [87, 107]. Regarding ERα, the AF-1 domain is actively involved in gene expression induced by agonists whereas the AF-1 domain of ERβ acts very weakly [108, 109].

1.2.2 Molecular mechanism of ERs

ERs regulate target gene expression through distinct pathways (Figure 3). The first one is the classical pathway of ER, ER is activated by ligands followed by dimer formation and direct binding to specific DNA sequence (ERE) located in or near the promoters of target genes through the DBD of ER, resulting in the recruitment of different transcriptional coregulators (coactivators or corepressors) to form a complex, which is responsible for the recruitment of transcriptional machinery, the modulation of chromatin structure and regulation of target gene expression [103]. Second, estrogen also modulates the expression of genes lacking ERE-like sequences, by a tethering mechanism, in which ligand-activated ER interacts with DNA indirectly via interacting with other transcription factors at their respective response elements, such as activating protein 1 (AP-1), stimulating protein-1 (Sp-1), nuclear factor-κB (NF-κB) as some examples [110, 111]. Furthermore, through the non-genomic pathway, membrane-localized ER can elicit a rapid response to ligands leading to activation of signaling transduction pathway in cytoplasm, such as PI3K/MAPK signaling pathway [87, 110, 111]. Additionally, ERs can regulate target gene expression in a ligand-independent manner, in which ER binds DNA directly or indirectly following ER activation through phosphorylation by growth factor signaling and other protein kinases [112, 113].

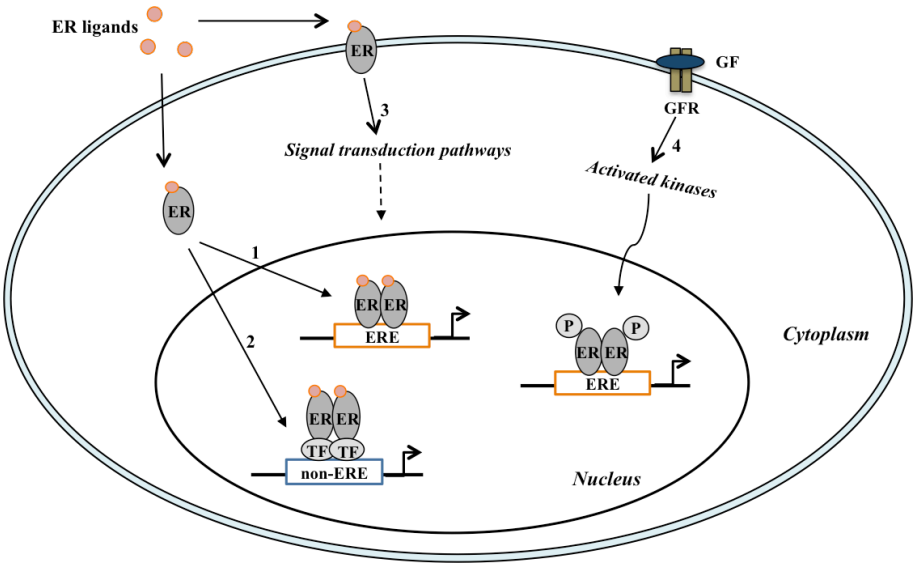

Figure 3. Simple model of mechanism of ER signaling. No. 1 is a classical pathway, ligand-activated ERs directly bind to specific DNA sequence, such as ERE; No. 2 is the tethered pathway, ligand-activated ERs interact with DNA indirectly via tethering with other transcription factors; No. 3 is the non-genomic pathway, membrane-localized ER activated by ligands results in activation of signaling transduction pathway in cytoplasm; No. 4 is the ligand-independent signaling, which is induced by membrane receptor signaling, such as growth factor (GF), with the result of phosphorylation of ER leading to the activation of ER binding with ERE.

Based on the current literature of ER transcriptome, E2-regulated gene expression is largely unique to each ER subtype, with distinct signaling pathways for ERα and/or ERβ, respectively [93, 114-117]. The comprehensive genome-wide mapping of ER DNA binding

regions indicates substantial overlap of two ERs binding sites, while regions bound by ERα have distinct properties compared with ERβ binding regions, including genome landscape, sequence features and conservation [93, 118, 119].

1.2.3 The role of ERs in breast cancers

Comparing with ERβ, ERα is a 'classic' and well-characterized ER. In normal mammary glands, the proportion of ERα-positive cells is generally low (10-20%) while the proportion increases in proliferative benign disease and low-grade ductal carcinoma in situ (DCIS) [103, 120]. *In vitro* studies also show that ERα-positive cell lines are dependent on estrogen for cell growth [121]. On the other hand, the expression of ERα is one of the indicators of hormone-dependent tumor growth. More than 50% breast cancers overexpress ERα and approximate 75% of these are estrogen-dependent at diagnosis [122]. The studies of the correlation between ERα expression status and the outcomes of breast cancer patients have indicated that the expression of ERα is considered to be a good indicator for endocrine therapy and breast cancer survival [123]. However, ERα status is not considered to be a perfect marker for responsiveness to anti-estrogens [114]. Because only 70% of ERα-positive breast cancer cases respond to tamoxifen and, interestingly, about 5-10% of the ERα-negative breast tumors are sensitive to tamoxifen treatment [116, 124].

Some studies have shown that ERβ could be a therapeutic target for those who have no response to tamoxifen and ERα-negative breast cancer patients [116, 125]. ERβ could be expressed in either ERα-positive or -negative breast tumors [120, 126]. In general, some studies indicate that ERβ may play a role as a tumor suppressor and may increase the sensitivity of ERα-positive breast tumors to tamoxifen [127-130]. However, in ERα-negative breast cancer tissue and cells, ERβ exhibits pro-growth and pro-survival activity [131]. Moreover, ERβ1 (wild-type ERβ) coexists with four ERβ variants (designated ERβ2 to ERβ5) that complicate elucidation of their physiological role and involvement in ER carcinogenesis [132]. ERβ1 is the one fully functional variant. Generally, it has been described that the expression of ERβ1 is downregulated or absent from high-grade breast tumors. However, some large cohort studies report no correlation between ERβ1 and clinical parameters, which suggests that ERβ1 is not a prognostic or predicting biomarker for breast cancer [116, 126]. Thus, the exact role of ERβ in human breast cancer remains unclear. The controversial roles of ERβ could be due to the lack of specific ERβ antibody, and different antibodies have been used in various labs. To develop highly selective and widely-used anti-ERβ antibodies remains a main challenge. One recent study has shown that only one anti-ERβ antibody (monoclonal PPZ0506) is selective for ERβ among 13 tested anti-ERβ antibodies [133].

1.3 ACTIVATOR PROTEIN-1

Activator protein-1 (AP-1) is a transcriptional factor which was first identified in 1987, and was found to bind to specific sequence of *cis*-control element of human metallothionein (hMTIIA) promoter and also binds to the enhancer region of simian virus 40 (SV40) [134]. The same year, the specific sequence, 5'-TGAG/CTCA-3', bound by AP-1 was discovered in

the promoters of hMTIIA and SV40, and also that the tumor promoter 12-O-tetradecanoyl phorbol 13-acetate (TPA) could strongly induce binding of AP-1 to this sequence, and thus this sequence was called TPA-response elements (TRE) [135, 136].

AP-1 is a dimeric complex comprising several protein families. The Jun proteins (c-Jun, JunB, JunD) and Fos proteins (c-Fos, FosB, Fra-1, Fra-2) are the major and early identified components of AP-1 proteins [137]. According to the specificity of DNA-sequence and heterodimerization with Jun or Fos proteins, some basic leucine zippers (bZIP) proteins are also included to the AP-1 protein family, such as activating transcription factor (ATF)/cAMP-responsive element-binding proteins (CREB) (ATF1, ATF2, ATF3/LRF1, ATF4, ATF5, ATF6a/b, ATF7, B-ATF, ATFa0 and CREB), musculoaponeurotic fibrosarcoma (MAF) (v-Maf, c-Maf, MafB, MafF, MafG, MafK and Nrl) and Jun-dimerizing partners (JDPs) protein families (JDP1/2) [138-144].

As AP-1 proteins belong to basic leucine zippers (bZIP) family, their protein structures contain a leucine zipper domain and a basic DNA-binding domain. The leucine zipper domain is required for the formation of homo- or heterodimers among various bZIP proteins. The DNA-binding domain is a known protein-DNA recognition motif and responsible for nuclear localization and DNA binding. Moreover, the specificity and stability of dimers formed by a variety of AP-1 proteins are also varied based on the composition of the leucine zipper [145, 146] (Figure 4). Except leucine zipper domain and DNA-binding domain, AP-1 proteins also contain two other domains, the docking sites and the transactivation domain [147] (Figure 5 and 6).

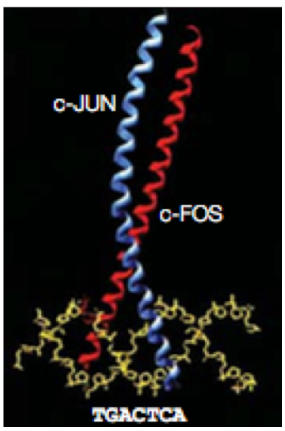

Figure 4. The bZIP domain on the DNA where AP-1 binds in form of an X-shaped heterodimer, resulting in an α-helical structure [148]. The bZIP domain of Jun and Fos is shown in blue and red, respectively. The DNA backbone is shown in yellow. Reprinted from Nat Rev Cancer, Nov 1, 2003, Vol 3, Issue 11, p859-68, Robert Eferl et al., AP-1: a double-edged sword in tumorigenesis [146], Copyright 2003, with permission from Springer Nature (license number: 4665851267602).

AP-1 regulates gene transcription through interaction with two possible elements, TRE and CRE (cAMP response elements, 5'-TGACGTCA-3'), via a bZIP domain [139, 149]. The only difference between TRE and CRE is one nucleotide in the middle [149, 150].

Jun proteins not only form homodimers but also form heterodimers with other members of AP-1 proteins. ATF proteins can also form homodimers [140]. However, Fos proteins do not have the ability to interact with DNA only by forming stable heterodimers with Jun proteins. For instance, previous studies have shown that the homodimer of c-Fos was found to be unstable *in vitro* and assumed that it could not exist in live cells [151, 152]. Interestingly, a recent study reported the evidence for the existence of stable c-Fos homodimers in live cells [153], but it is still not clear whether the c-Fos homodimer is functional or not. Furthermore, *in vitro* studies have shown that Jun/ Fos heterodimers have higher stability than Jun homodimers [150, 154].

With the three members of Jun proteins, the Jun or Fos protein families can form 18 different homo- and heterodimers, and these dimers have the highest affinity to TRE and slightly lower affinity to CRE [151]. Jun/ATF heterodimers or ATF homodimers prefer to bind to CRE [155]. MAF proteins recognize another longer palindromic sequence, MAF-recognition element (MARE), which consists of TRE or CRE. Jun/MAF and Fos/MAF heterodimers prefer to interact with the sequence with half part of TRE or CRE and half part of MARE [143, 151, 156]. Thus, the promoter-binding specificity and affinity of AP-1 are affected by the alteration of AP-1 dimer composition.

Moreover, AP-1 transcription factors have essential effects on various cellular processes, such as proliferation, differentiation, apoptosis and inflammation [146, 147, 154]. A variety of cellular stimulation, including growth factors, cytokines, UV radiation, bacterial and viral infection and cellular stress, is able to regulate AP-1 transcriptional activity [147]. The dimer composition is critical to regulate activity of AP-1 proteins resulting in regulation of specific target genes [142]. For example, some studies found that the ratio of c-Jun/c-Fos and c-Jun/ATF2 dimers existing in cells is able to determine the cellular response to apoptotic or oncogenic stimuli [157, 158].

1.3.1 The expression and functions of Jun family

The proto-oncogene c-Jun was first isolated and identified as a human counterpart of the viral homolog in the avian sarcoma virus 17 encoded oncogene, v-Jun, by Vogt and colleagues [137, 150]. Then the group of Dan Nathans identified other two Jun family members (JunB and JunD) [159, 160] (Figure 5). The gene of c-Jun is intronless, located on chromosome 1 (1p32-p31) [161] while JunB and JunD both maps to chromosome 19.

c-Jun gene is expressed at low levels in many cell types, but it is rapidly induced by exposure to different extra-cellular signals. There is a feedback loop in AP-1 activating c-Jun promoter, in which c-Jun can auto-regulate its own expression [162]. Moreover, similar to other AP-1 family members, the activation of c-Jun is also regulated by post-translational regulation, especially phosphorylation via c-Jun N-terminal kinases (JNKs) or the kinases ERK1, ERK2

and GSK3β [142] (Figure 5). JNKs have been demonstrated to be important for c-Jun phosphorylation in response to cellular stress and for a basal level of c-Jun expression [163]. JNKs phosphorylate c-Jun on Ser 63/73 and Thr 91/93 residues at its transactivation domain leading to regulation of the transactivation activity [164]. In addition, phosphorylation of c-Jun by JNKs can protect c-Jun from degradation by ubiquitination and hence increase the half-life of the protein [155, 165].

Although the structures of JunB and JunD are similar to c-Jun, JunD lacks the JNK docking site while JunB does not have JNK phosphorylation sites [166]. Thus, JNKs phosphorylate JunD less efficiently and the phosphorylation activation of JunB is independent of JNKs.

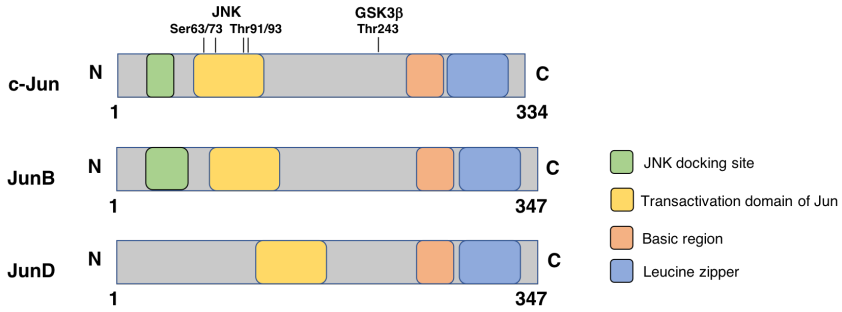

Figure 5. Schematic presentation of the structure of Jun protein family. Jun proteins contain several domains, including bZIP domain (basic region and leucine zipper), transactivation domains and docking sites for several kinases, while JunD lacks the JNK docking site.

The three members of the Jun protein family are distinct in their biological function [146, 155]. Previous mouse genetic studies have indicated that c-Jun and JunB are essential for normal mouse embryonic development. The c-Jun-deficient mice die between embryonic days E12.5 and E13.5 [167]. Lack of JunB causes embryonic lethality between E8.5 and E10.0 [168]. However, JunD -/- mice are viable and appear healthy [119]. Moreover, JunB can rescue c-Jun-/- mice embryo phenotypes dose-dependently and also partially replace the role of c-Jun in the regulation of c-Jun gene expression [169].

Overexpression of Jun family members is found in many human cancers [142, 147, 170]. The expression of c-Jun plays an important role in tumor development, such as in liver, skin and breast tumors [146, 171]. Generally, c-Jun exhibits oncogenic functions. For example, it has been reported that c-Jun positively regulates the growth and angiogenesis of solid squamous cell carcinomas [172], and c-Jun works together with p53 to protect liver tumor cells from apoptosis [173]. Moreover, c-Jun is involved in tumor cell migration, invasion and epithelial-mesenchymal transition (EMT) [174, 175]. However, JunB has been shown to exhibit dual roles [155]. It has been shown that JunB is overexpressed and involved in human cancers. For instance, a study suggests that JunB cooperates with c-Jun in mediating TGFβ-induced genes associated with invasion and cancer progression [176]. In contrast, JunB as a tumor suppressor has been shown in several *in vivo* studies [177, 178] and also acts oppositely to the role of c-Jun in cell proliferation [179, 180]. Furthermore, the biological activities of JunD are often opposite and antagonistic to c-Jun activities [155]. A study found that in prostate

cancer cells, overexpression of JunD increases the proliferation rate while overexpression of c-Jun and JunB decreases the proliferation rate [181].

1.3.2 The expression and functions of Fos family

The Fos protein family consists of four members, including c-Fos, FosB, Fos related antigen-1 (FosL1 or Fra-1) and Fos related antigen-2 (FosL2 or Fra-2) (Figure 6). The proto-oncogene c-Fos was discovered as the human homolog of the retroviral oncogene v-Fos in the osteosarcoma virus and its expression can induce cellular transformation in rat fibroblasts [182, 183]. FosB gene was first identified with growth factor stimulation and indicated that FosB interacts with c-Jun and JunB proteins result in increase of their DNA binding activity [184]. Fra-1 and Fra-2 genes were isolated by screening human cDNA library from serum-stimulated rat fibroblasts [185, 186].

c-Fos and Fra-1 are the most studied Fos proteins, and the former one is the prototype of Fos family. Except common domains (bZIP domain and DNA binding domain) shared among AP-1 proteins, c-Fos and FosB proteins have a C-terminal transactivation domain (Figure 6). This domain functions in transcriptional activation, stabilizing the pre-initiation complex and facilitating its assembly, and it is critical for the transformation capacity of Fos proteins [187, 188]. However, Fra-1 and Fra-2 lack this domain. Due to Fos proteins having no ability to form homodimers, they need the dimerization partners, Jun proteins, which mainly influence their role in gene activation [144, 189]. Like Jun family members, c-Fos and FosB are also immediate early genes which have rapid and transient transcriptional activation in response to mitogenic stimulation or cellular stress [190, 191]. However, the increase of Fra-1 and Fra-2 expression is delayed and stable compared with that of c-Fos and FosB [187].

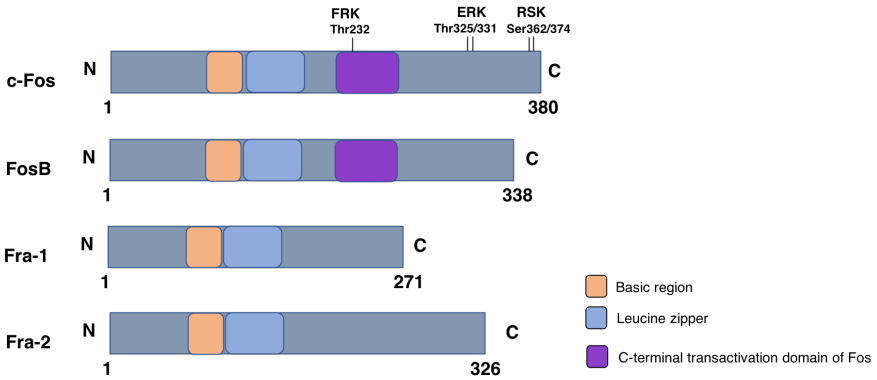

Figure 6. Schematic presentation of the structure of Fos protein family. All Fos proteins contain bZIP domain (basic region and leucine zipper). c-Fos and FosB proteins also have transactivation domains at C-terminal.

Fra-1 knockout mice show embryonic lethality between E10.0 and E10.5, and Fra-2 knockout mice die shortly after birth, indicating the important role of these two proteins in mouse development [192, 193]. Several other mouse model studies have shown that Fos family proteins play an important role in normal tissue and tumor development [146, 154, 194]. For example, overexpression of Fra-1 and Fra-2 in transgenic mice results in the development of

lung tumors and epithelial tumors, respectively [195]. Moreover, c-Fos and Fra-1 are frequently overexpressed in tumor cells or tissue [195-197]. Further studies show that c-Fos, Fra1 and Fra2 might involve in the invasion process of breast cancer [154, 198, 199]. FosB has been shown to be highly expressed in normal mammary epithelia but downregulated in poorly differentiated mammary carcinomas [198, 199]. Recent evidences have shown that Fra-1 is a key regulator to drive EMT and hence increases invasive and metastatic capabilities of tumor cells [200]. However, there are some studies indicating that c-Fos has a tumor suppressor activity in various cancer types, such as gastric carcinoma, hepatocellular tumorigenesis and epithelial ovarian carcinoma [201-203].

The activity of Fos family proteins is also modulated by phosphorylation via different kinases, including MAPK, RSK, ERK, PKA or PKC, to influence protein stability, DNA-binding activity, transactivation and transforming activity [154, 204, 205]. c-Fos and Fra-1 are degraded through the N-terminal destabilizer domains by ubiquitin dependent mechanisms and they also, like Fra2 and FosB, undergo a ubiquitin-independent degradation by proteasome [206-208].

1.3.3 The role of AP-1 in breast cancer

AP-1 is a key component of various signal transduction pathways, and it regulates various cellular events including proliferation, differentiation, survival, angiogenesis, migration and invasion [146]. The activity of AP-1 is enhanced in numerous human tumor types, thereby playing a key role in tumorigenesis [209].

In breast cancers, AP-1 family members are important regulators of cell growth through multiple mechanisms. AP-1 blockade inhibits breast cancer cell growth induced by many factors, such as estrogen, epidermal growth factor (EGF), heregulin (HRG) and insulin-like growth factors (IGFs) [210]. In line with this, an *in vivo* study reported that blocking AP-1 in established breast tumors suppresses its growth in nude mice [210]. Furthermore, a study indicated that AP-1 regulates the expression of cyclin D and E2F and their target genes to mediate the cell cycle and cell proliferation of breast cancer cells [211, 212].

In addition, it has been shown that AP-1 is overexpressed in the aggressive subtype of breast cancer, such as TNBC/basal-like, compared with ERα-positive breast cancer [170, 213, 214]. High AP-1 expression is associated with poor prognosis of breast cancer. For example, analysis of cDNA microarray data including 197 breast cancer patients showed that high expression of Fra-1 significantly correlates with shorter overall survival and higher percentage of lung metastasis in ERα-positive breast cancer patients [215]. Moreover, a role for Fra-1 or c-Jun in promoting breast cancer cell metastasis *in vivo* has been demonstrated in a zebrafish tumor xenograft model [214], and Fra-1 has been identified as a key regulator involved in the process of metastasis in rodent model systems [216]. In addition, it has been shown that AP-1 is a key regulator of inflammation-induced cancer progression and also involved in the inflammation-induced EMT in TNBC [217, 218].

Clinically, it has been shown that transcriptional responses of AP-1 were increased in tamoxifen resistant ERα-positive breast cancer [219]. Additionally, tamoxifen resistance is also associated with increased JNK activity [220]. Furthermore, the ER/AP-1 cross-talk plays an important role in tamoxifen resistant breast cancer. The stress-related kinases and growth factor receptors (GFRs), both of which are upstream of AP-1, are implicated in breast cancer with endocrine resistance [221]. A previous study reported that tamoxifen was a potent transcriptional activator of ERβ at an AP-1 site [222]. Moreover, reprogramming of ERα nuclear genomic function through its binding to AP-1 sites might be a feature of endocrine therapy resistance [221]. Together, AP-1 and/or its signaling pathways could serve as entry points for targeted therapies for breast cancers.

Several compounds, including synthetic inhibitor and bioactive compounds, have been identified based on the mechanism of AP-1 inhibition [209, 223]. T-5224 is a selective AP-1 inhibitor initially investigated for the treatment of rheumatoid arthritis in phase II human clinical trials. However, this was ceased for unreported reasons in 2008 [144]. T-5224 shows no effect on AP-1 protein expression over several time points but specific inhibition of DNA binding activity of c-Fos/c-Jun, whereas other transcription factors like MyoD, Sp-1, NF-κB/p65 remain unaffected [224]. Nevertheless, most currently identified compounds lack specificity and thus today no effective inhibitors against AP-1 have been approved for application in the clinics [223].

1.4 TRANSFORMING GROWTH FACTOR β INDUCED (TGFBI) PROTEIN AND BREAST CANCER

TGFBI protein is a transforming growth factor β inducible secreted extracellular matrix (ECM) protein. This protein was originally known as βig-h3, because its gene (TGF-β-induced gene-human, clone 3, βig-h3) was first identified from a cDNA library of a human lung adenocarcinoma cell line A549 treated with TGF-β [225, 226]. This gene is located on the chromosome 5q31 and encodes a 683-amino-acids, and the predicted molecular mass of TGFBI secreted form is 68kDa. TGFBI protein contains an N-terminal secretory signal peptide (SP), a cysteine-rich domain (CRD), four consecutive fasciclin-1 (FAS1) repeats, which contain several known integrin-binding motifs (e.g. NKDIL, YH18, and EPDIM), and a C-terminal Arg-Gly-Asp (RGD) integrin-binding motif [227, 228].

TGFBI protein has been detected in most normal human tissues [229]. Although TGFBI is a downstream component of TGF-β signaling pathway, its expression is regulated not only by TGF-β, but also by other factors and mechanisms, such as autophagy [230], microRNAs [231], interleukin (IL)-1β [232], IL-4 [233], tumor necrosis factor α (TNFα) [232], cancer-associated fibroblasts (CAFs) [234], and high glucose concentrations [235, 236]. TGFBI mediates cell adhesion, migration, proliferation, apoptosis, and angiogenesis through interaction with several ECM molecules (e.g. collagen, fibronectin, and laminin) and integrins (e.g. α1β1, α3β1, αvβ3, and αvβ5) [228, 235, 237-240]. The integrins are the major TGFBI cell surface receptors identified to date [227]. In addition, abnormal expressions of

TGFBI are associated with various diseases, including corneal disorders [241], diabetes [242] and many types of cancers [228, 236].

The functions of TGFBI are dependent on the tumor cell type and microenvironment, and it has dual effects, including acting as tumor suppressor or tumor promoter [228]. Studying TGFBI knockout mice showed that lack of TGFBI displays a higher incidence of spontaneous tumor growth and chemical carcinogen-induced skin tumors compared with wild-type mice, suggesting that TGFBI acts as a tumor-suppressor [243]. In contrast, a recent study indicated that the role of TGFBI in gastrointestinal tract is as a tumor promoter, and the overexpression of TGFBI in mice induces spontaneous tumors [244].

Regarding the tumor suppressor function of TGFBI, several studies reported that down-regulation of TGFBI was identified in various tumors cells and correlated highly with its promoter hypermethylation [245, 246]. For example, the expression level of TGFBI protein is reduced and only trace amount is detected in breast tumor cell lines [229, 247]. In a study of Bingyan and colleagues [247], *in vitro* and *in vivo* experiments identified the suppressive role of TGFBI in the development of breast cancer cells via possible mechanisms, including suppression of cell proliferation, delaying of G1-S phase transition and induction of senescence. Moreover, it has been shown that TGFBI induces adhesion to ECM proteins, and inhibits metastatic ability both *in vitro* and *in vivo* [229].

On the other hand, there are increasing data indicating that TGFBI exhibits a tumor-promoting function. Overexpression of TGFBI has been noted in various tumor tissues and cell lines [227]. For example, recombinant TGFBI promotes mobility and invasiveness of ovarian carcinoma cells [248]. Furthermore, the increased TGFBI expression has been related to the aggressiveness of tumors [228, 240, 249]. Additionally, a gene expression profile analysis identified TGFBI mRNA levels to be relatively increased in two highly invasive breast cancer cell lines, including BT549 and Hs578T [250].

Previous studies have shown that the expression of TGFBI appeared to induce paclitaxel sensitization in ovarian cancer [251]. However, a microarray-based gene expression analysis identified high expression of TGFBI involved in topotecan-resistant ovarian cancer [252]. Moreover, it has been reported that TGFBI might be associated with Lapatinib resistance in HER2-positive breast cancer cell lines [253]. On the other hand, epigenetic silencing of TGFBI by DNA methylation has been found to contribute to the trastuzumab resistance in HER2-positive cell models [254]. Taken together, TGFBI can function as a chemo-sensitizer or a risk of chemo-resistance in various cancers.

1.5 COREGULATORS OF TRANSCRIPTION FACTORS

The activity of transcription factors (TFs) and distinct expression patterns of genes regulated by TFs in different tissues are controlled by another class of molecules, known as the coregulators [255]. A majority of coregulators organized in large multi-protein complexes are recruited to the genome by DNA-binding TFs and thereby regulate (activate or repress) the transcription of specific genes [256, 257]. Transcriptional coregulators that enhance the

transcription activity are referred to as coactivators while those that repress the transcription activity are known as corepressors. Coregulators exhibit protein-protein interaction with TFs that bind to specific genomic loci [255]. Each complex of coregulators can be recruited to various TFs, and each TF can recruit many different complexes of coregulators [257].

Many coregulators interact with nuclear receptors, such as ER, in a ligand- and AF-2-dependent manner [258]. Most coactivators are recruited to ER through a conserved motif 'NR box' with the sequence LXXLL (X, any amino acid; L, Leucine) [259]. For instance, steroid receptor coactivators (SRCs)/p160 are the primary coactivators of ERα and they contain NR box that interact with AF-2 domain to recruit to ER, followed by recruiting the secondary coactivator, including histone acetyltransferase (HAT), coactivator-associated arginine methyltransferase 1 (CARM1), CREB-binding protein (CBP)/p300, ATP-dependent chromatin remodeling complexes and many others [260].

Additionally, transcription corepressors interact with ER through LxxH/IIxxxI/L motif (CoRNR box; I, Isoleucine; H, Histidine) to inhibit its transcriptional activity [261, 262]. For example, Peohibitin (PHB) functions as a corepressor of ERα to inhibit ERα-mediated transcriptional activation in breast cancer cells and also interacts with histone deacetylases 1 (HDAC1) [263]. Additionally, corepressors also exhibit negative function through a direct interaction with unliganded ER or by competing with coactivators for ER binding, such as receptor-interacting 140 (RIP140) that can antagonize coactivator SRC-1 and also recruit HDACs [113, 260]. ERs could also associate with other nuclear receptor corepressors, such as nuclear corepressor (N-CoR) [264, 265].

HATs or HDACs are one of the most studied enzymes. They form part of coregulator complexes and have essential roles in modifying chromatin. CBP/p300 interacts with almost all TFs and regulates gene expression by opening chromatin structure at the target gene promoter through the HAT activity [266]. Moreover, SRC-1 can form a complex with CBP/p300 that coactivates the AP-1 mediated transactivation [267]. CREB-related transcription coactivator 1 (CRTC1) acts as a coactivator through direct interaction with the bZIP regions of c-Jun and c-Fos to control AP-1-mediated transcriptional response to TPA [268]. In addition, several AP-1 target genes were proved to be occupied by N-CoR/SMRT corepressor complexes under basal conditions [269]. Many factors initially identified as nuclear receptor coregulators have been demonstrated to act as AP-1 coregulators [269].

Thus, the full characterization of the nature and composition of transcription factor interacting protein complexes in cancer cells will provide essential information to understand how they control target gene specificity, cellular signaling and phenotypes, ultimately to identify their potential roles as therapeutic targets.

1.5.1 DDX5 and breast cancer

DDX5 (p68) is a member of the DEAD (Asp-Glu-Ala-Asp)-box family belonging to the RNA helicases. DDX5 was first identified as a nuclear antigen that cross-reacts with an antibody against the T-antigen of Simian Virus 40 [270]. The DEAD-box proteins contain a

helicase core that includes two domains at N- and C-terminal, respectively [271, 272]. The N-terminal domain is comprised of motifs Q, I, Ia, Ib, II and III, and the C-terminal domain contains IV, V and VI motifs [273]. The nine conserved regions harbor for RNA binding, ATP binding and hydrolysis, and intermolecular interaction [274]. DDX5 shares 90% homology of the helicase core with another DEAD-box protein, DDX17 (p72), and these two proteins can form heterodimers in cells [275].

DDX5 protein is 69kDa with 614 amino acids encoded by DDX5 gene which is located on chromosome 17q23. The expression of DDX5 is ubiquitous in human tissues and plays multiple functions, including ATPase activity, RNA unwinding, transcription and RNA processing activities [273, 276, 277]. Moreover, DDX5 is involved in cell growth, early development and maturation of some organs [278-280]. Additionally, several experimental results reveal examples of diseases associated with DDX5, including obesity [281, 282], Down syndrome [283], myotonic dystrophies [284] and especially cancer [285, 286].

With regards of cancers, DDX5 has a critical role in cancer development [286, 287]. The abnormal expression of DDX5 was identified in various cancers, including breast cancer [288], lung cancer [289], colorectal cancer [290, 291], colon cancer [292, 293], multiple myeloma [294], cutaneous squamous cell carcinoma [295], leukemia [296, 297] and head and neck squamous cell carcinoma [298]. Accordingly, DDX5 acts as a transcriptional coregulator for several cancer-associated TFs, such as AR [299], ERα [272, 300], tumor suppressor p53 [301], β-catenin [302, 303], MyoD [304], Runx2 [305], Notch transcriptional activation complex [306], NF-κB, and signal transducer and activator of transcription 3 (STAT3) [306]. In addition, some studies have indicated that DDX5 also participates in the transcription initiation [301, 307, 308].

DDX5 is frequently overexpressed in breast cancer, particularly in higher grade and poor prognosis breast tumors [300]. As a transcriptional coactivator of ERα, DDX5/DDX17 act as the key regulators of estrogen-signaling pathways by controlling both upstream and downstream of the ERα at transcriptional and splicing level [309, 310].

Moreover, DDX5, a coactivator of β-catenin, functions in the expression of TCF4 mediated by Wnt signaling, which is a tumor promoting pathway, in breast cancer cells [273, 311]. The study of Guturi and colleagues [302] showed that in breast cancer, β-catenin/TCF4 and DDX5 constitute positive feedback loop that are essential for Wnt/β-catenin-signaling involved in tumorigenesis. On the other hand, β-catenin/TCF4 upregulates DDX5 expression leading to EMT in breast cancer cells [302].

STAT3 is functionally active in numerous cancers, particularly in half of the breast cancers, and has been confirmed to be constitutively active in TNBC [312, 313]. DDX5 has been reported to be a coactivator of STAT3 and upregulate the downstream genes of STAT3 which are associated with a wide range of tumorigenic processes, such as cellular proliferation, survival, invasion and angiogenesis [306]. DDX5 also interacts with long non-coding RNA (lncRNA), such as LOC284454, to modulate cancer-related pathways and

pathology of breast cancer, such as focal adhesion and cell migration [314]. Furthermore, post-translational modification of DDX5 can regulate the coactivation effects, especially phosphorylation of DDX5 at tyrosine residues has been shown to be associated with abnormal cell proliferation and cancer development [315].

In total, according to the above-described functions of DDX5 in breast cancer, DDX5 is considered to be an excellent candidate as therapeutic target [316]. More than that, the depletion of DDX5 enhances the sensitivity of HER2-positive breast cancer cells to trastuzumab [317]. A study has suggested that small molecule inhibitors could selectively target the activity of DEAD-box family members [286, 318]. The phosphorylation of DDX5 at Tyr593 residue was previously identified only in transformed cancer cells, but not in normal cells [287]. Therefore, phosphorylated-DDX5 (p-DDX5) could be specifically targeted by anticancer molecule exhibiting strong growth inhibition, such as RX-5902 (Supinoxin), which binds directly to p-DDX5 in cancer cells to inhibit the interaction between p-DDX5 and β-catenin pathway resulting in blocking the β-catenin pathway and its downstream genes (e.g. c-Jun, c-Myc and cyclin D1) [287, 319]. RX-5902 induced G2/M arrest and apoptosis in TNBC cells, and had additive effects of anti-tumor *in vivo*, currently, a phase 2 clinical trial in TNBC is ongoing (https://clinicaltrials.gov/ct2/show/NCT02003092) [320]. Further studies on the precise mechanism of DDX5 in cancer progression are essential to development of novel therapeutic approaches.

2 AIMS OF THE THESIS

The overall aim of this thesis is to comprehensively decipher the role of the ER and AP-1 transcription factors in breast cancer, using functional genomics technologies that today can identify the cistrome, transcriptome and proteome at an unprecedented detail, thereby, this could further provide information for developing novel therapeutic strategies for breast cancer. In particular, the three specific aims were:

I. To explore the genome-wide overlap in DNA binding profiles of two transcription factors, ER and AP-1, and to understand their coordinated interaction at the genome level.

II. To identify the chromatin interactome of Fra-1 in TNBC cells and hence to detect some novel coregulators of Fra-1.

III. To provide a novel and valuable resource to further complement the knowledge of ERα or ERβ uniquely mediated gene transcription in ERα-positive breast cancer cells.

3 METHODOLOGICAL CONSIDERATIONS

The details of materials and methods are described in each constituent study. In this section, discussion of considerations and limitations of used methods are described as below.

3.1 CELL LINES

Cell lines are easy to handle, and they also could grow infinitely and provide a consistent sample. Therefore, in cancer research, a majority of *in vitro* models are cell lines. However, there are several limitations for cell lines. Due to the extended period of cell culture, serial passage of cell lines can cause variation of genotype and phenotype and genetic drift, as well as lead to heterogeneity in cultures at a single point in time, which could cause the various results detected from the same subtypes and even the same cell line is different between different labs [321].

In this thesis, two groups of cell lines were used, including TNBC cells and non-TNBC cells, which were purchased from American Type Culture Collection (ATCC). In Paper I, we used MCF7 cells with inducible Tet-off system (Clontech) expressing AP-1 protein, because AP-1 components, such as c-Jun and Fra-1, express at very low levels in available cell lines of ERα positive breast cancer [214]. We also used MCF7 cells with Tet-off induced expression of ERβ in Paper III, due to the fact that there were no ERα-positive breast cancer cell lines expressing significant levels of ERβ. Furthermore, we performed gene editing using CRISPR/Cas9 system on this cell model. We used four types of TNBC cells (BT549, Hs578T, MDA-MB-157 and MDA-MB-231) and eight non-TNBC cells (MCF7, T47D, MDA-MB-175, MDA-MB-453, ZR-751, CAMA-1, HCC1569 and SK-BR-3). W.G. Coutinho and E.Y. Lasfargues isolated BT549 cells in the year of 1978. Because BT549 has high expression of AP-1 and transfection is easier compared with other TNBC cells, BT549 was chosen to be the representative cell model to study TNBC.

3.2 TET-OFF GENE EXPRESSION SYSTEM

Tet gene expression systems are used to regulate the activity of gene in eukaryotic cells [322]. Tet-off and Tet-on cell lines are commercial and provide a ready way to study the gene of interest at high expression level. In each system, there are two critical components, including tetracycline (Tet)-controlled transcription factor and response plasmid expressing interest gene.

In Tet-off system, tetracycline-controlled transactivator (tTA) is from the fusion of E. coli Tet repressor protein (TetR) and Herpes simplex virus VP16 activation domain. The pTet-off regulator plasmid encodes tTA and the target gene expression of tTA is regulated under transcriptional control of a Tet-responsive promoter element. In the absence of Tet or doxycycline (Dox), TetR part of tTA will bind to the Tet operator sequences (tetO) in tetracycline-responsive element (TRE) and further active the gene expression. However, when Tet or Dox is present, TetR will bind to Tet other than tetO sequences to inactive the gene expression (Figure 7A). Tet-on system is based on a reverse tTA (rtTA) generated by

altering four amino acids, which can only bind to tetO sequences in the TRE in the presence of Dox. Therefore, in tet-on system, the expression of the gene of interest is activated by rtTA only with Dox (Figure 7B).

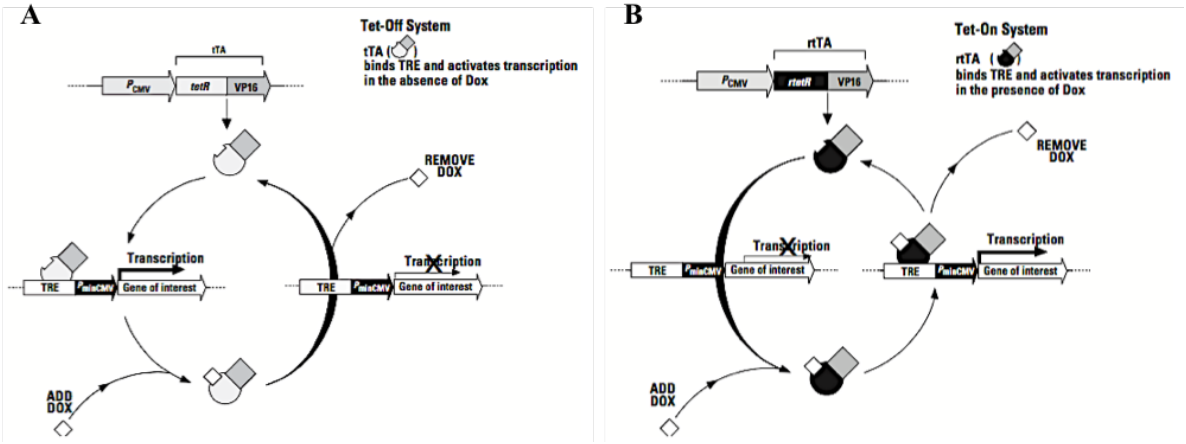

Figure 7. Schematic diagram of the Tet-off and Tet-on systems. The TRE locates at the upstream of the minimal immediate early promoter of cytomegalovirus (*P*minCMV), which keeps silent state without activation. A, In the Tet-off system, tTA binds to TRE without Tet or Dox that causes the activation of the gene transcription. B, The Tet-on system only responses to Dox. In the presence of Dox, rtTA can bind TRE and active transcription. From Tet-Off® and Tet-On® Gene Expression Systems User Manual (Clontech Laboratories, Inc®).

In this thesis, the genes of interest (AP-1 or ERβ) should be active, and only be turned off occasionally, so we used Tet-off gene expression system.

Regarding the response plasmid expressing gene of interest, we used pBI-EGFP plasmid, which can coexpress the genes of interest (AP-1 or ERβ) and enhanced green fluorescent protein (EGFP) controlled via bidirectional promoter. The expression of the target genes can by monitored by EGFP expression using fluorescence-activated cell sorting (FACS) or fluorescence microscopy. The relative expression levels of target genes can be inferred at the levels of EGFP expression in the absence or presence of Tet or Dox (Figure 8).

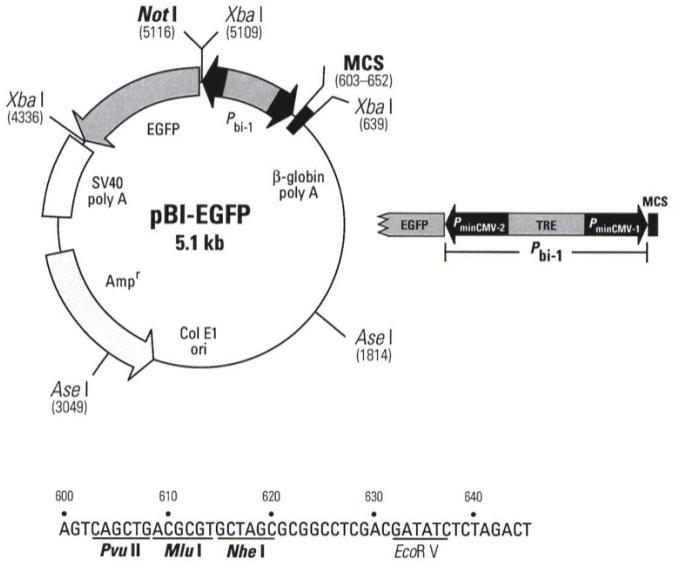

Figure 8. Restriction map of pBI-EGFP. pBI-EGFP vector contains a bidirectional tetracycline-response promoter (*P*bi-1). In the Tet-off and Tet-on systems, *P*bi-1 is responsive to tTA and rtTA regulatory proteins, respectively. AP-1 or ERβ cDNA was cloned into the Multiple Cloning Site (MCS). From pBI-EGFP vector information (Clontech Laboratories, Inc®).

3.3 CRISPR/CAS9-BASED GENE EDITING

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas is a microbial adaptive immune system. This system is used to cleave foreign genetic elements through RNA-guided nucleases. Among three identified types of CRISPR mechanisms, the type II system is the most studied and well characterized one, which can induce site-specific DNA cleavage by using noncoding RNAs to guide the Cas9 nuclease [323]. Through cellular DNA repair mechanisms, including the homology-directed repair (HDR) pathway or the non-homologous end joining DNA repair pathway (NHEJ), to repair this DNA damage (Figure 9).

The CRISPR/Cas9 system is a simple and precise RNA-programmable method which is applicated to regulate genome editing in a variety of mammalian cells and organism resulting in the gene knockins (via HDR) or knockouts (via insertion or deletion).

In this thesis, we deleted *ESR1* gene by NHEJ mutation pathway, resulting in insertions that disrupted the *ESR1* locus.

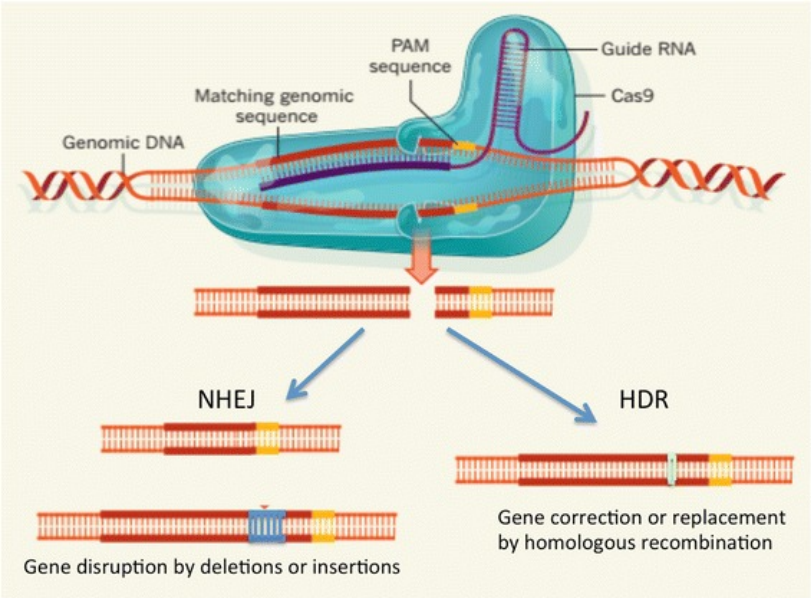

Figure 9. Schematic of CRISPR/Cas9-based gene editing. The Cas9 nuclease is targeted to genomic DNA sequence through a guide RNA. The guide RNA contains a 20-nt guide sequence that pairs with the DNA target, immediately upstream of the protospacer-associated motif (PAM). This mediates a double-strand break (DSB) 3 bp upstream of the PAM. The DSB can be subsequently repaired by NHEJ or HDR pathway. Reprinted from Mol Neurodegener, Vol 10, Zhuchi Tu et al., CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases [324], Copyright 2015, Used under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0).

3.4 GENE EXPRESSION ASSAYS

Gene expression can provide the information to interfere cellular signaling and cellular responses at an indicated time point. The following described assays were used in this thesis to target a single gene or at a genome-wide scale.

3.4.1 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a method to quantify gene expression in real time, which is also called real-time PCR. The amplification of DNA during PCR process can be detected in real time by using fluorescent reporter. The two major options of fluorescent reporter are intercalating dye and hydrolysis probe-based. The most common used intercalating dye is SYBR Green, which can bind to the DNA double helix to alter the dye structure and further make the dye to generate more fluorescence. Regarding the hydrolysis probes, e.g. Taqman probe, they are fluorescently labeled DNA oligonucleotides, a fluorescent reporter molecule at 5'end and a quencher molecule at 3' end, which could bind to the downstream of the primer specifically during the PCR process followed by fluorescing. In this thesis, we choose SYBR Green dye to perform qPCR due to low cost when detecting a variety of different genes and most of our interested genes are abundant. Additionally, by

measuring the PCR efficiency, we confirmed that the SYBR Green dye amplified a single product using a melting curve and obtained the similar results as Taqman probe [325].

qPCR is a very sensitive and powerful DNA analysis approach, it is ease of use and needs relatively short period of time for quantifying mRNA transcripts [326]. However, there are several limitations regarding qPCR. For instance, researchers need to acquire the information of the sequence of the target gene and the approach is a low throughput method.

3.4.2 High-throughput gene expression analysis

Gene expression microarray and RNA-sequencing (RNA-seq) are two main approaches to provide transcriptome-wide gene expression profiles for a sample.

Since the development and application of microarray during mid-1990s, which made the high throughput gene expression analysis possible [327, 328]. Microarray can detect nucleic acids in a sample by hybridization to probes on microchips, which is ease of sample preparation and low cost per sample. However, microarray assay should base on the existing knowledge of genome sequence, and its dynamic range of detection is limited caused by both background and saturation of signals [329]. With the rapid development of next-generation sequencing from early 2000s, the application of RNA-seq offers an alternative method for gene expression studies. RNA-seq can directly determine cDNA sequence based on simple counting of reads. Thus, it could overcome some limitations of microarrays. Moreover, RNA-seq has more applications, such as differential splicing analysis, detection of allele-specific expression and identification of novel transcripts [328]. The read depth is a key factor that influences the reliability of quantification of expression levels, and high variance is a character of low transcript abundances [330].

In the Paper I, we performed gene expression microarray by Affymetrix Clariom^{TM} D arrays, which is a next-generation transcriptome-level expression analysis tool offering a fast path to results. This assay gets unprecedented coverage of all known transcripts, regardless of abundance, available for human, mouse and rat. Moreover, it is the most comparable method to RNA-seq because it offers strand specific hybridization and is able to detect low expressing transcripts, but unlike sequencing. In Paper II and III, we performed RNA-seq using Illumina HIseq technology. DESeq 2 workflow was performed to analyze the differential gene expression.

3.5 CHROMATIN IMMUNOPRECIPITATION (CHIP)

ChIP assay was the critical assay in the first two papers, Paper I and II, included in this thesis. This assay is used for probing DNA-protein interactions and identification of the specific genomic location of associated proteins in a living cell. By crosslinking the DNA and proteins in live cells, researchers can get snapshot information for specific protein-DNA interactions and quantitate the interaction by qPCR. In addition, this assay can also combine with high-throughput sequence technology, such as ChIP-seq, to provide the interactions at genome-wide scale [331, 332]. Integrating cistrome data paralleled with transcriptome data is

a powerful and comprehensive analysis to find out the role of DNA binding of distinct factors on gene expression.

The reliability of ChIP experiment is mainly dependent on the quality and specificity of antibodies. In addition, the cell number and the range of chromatin fragments after sonication can also cause various results.

3.6 RAPID IMMUNOPRECIPITATION MASS SPECTROMETRY OF ENDOGENOUS PROTEINS (RIME)

To gain insight into the endogenous protein complex interacting transcription factor (in case Fra-1), we used RIME approach in Paper II, which was the critical and foundational experiment for the following study of Paper II. RIME was developed and optimized by Hisham Mohammed and colleagues [333] based on the strategy that combination of ChIP with mass spectrometry was applicated to study the composition of protein complexes associated with chromatin. Similar to regular ChIP described above, cells are crosslinked by formaldehyde, chromatin sheared by sonication and endogenous complexes immunoprecipitated by antibodies (Figure 10), followed by on-beads digestion, which can increase digestion efficiency and avoid antibody contamination. In addition, the assay has the ability of identifying endogenous protein complexes without engineering cells at either DNA or protein level.

The method is enough sensitive for investigating the high affinity and transient interactions of complexes formed by low-abundance proteins. Similar with the main limitation of ChIP, it needs high-affinity and high-specificity antibodies to get high-quality results.

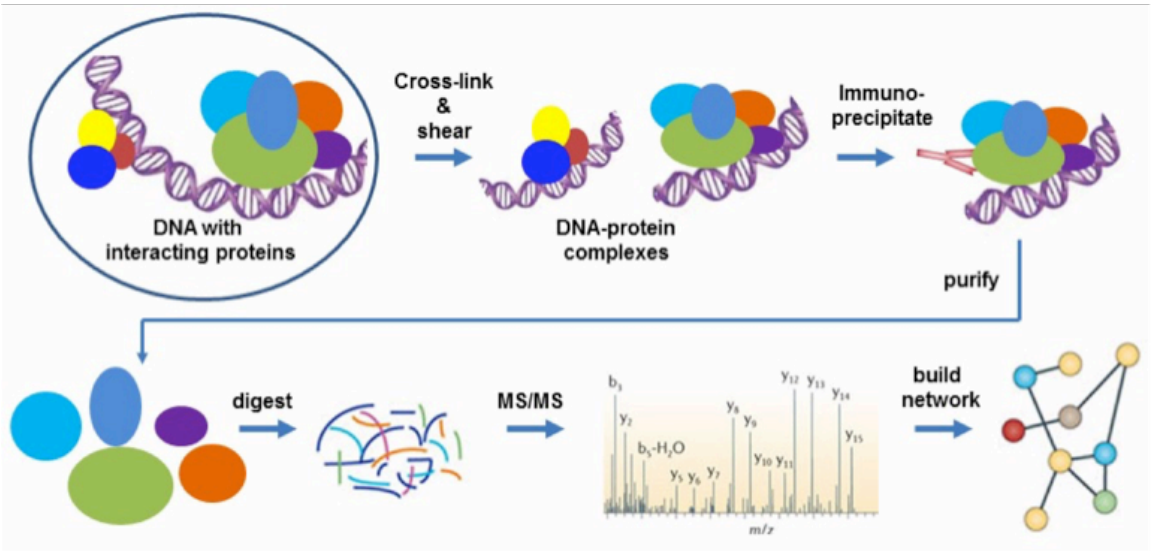

Figure 10. RIME workflow. Protein-protein interactions are crosslinked followed by sonication to shear chromatin. Subsequently Fra-1 complexes are immunoprecipitated with Fra-1 antibody and Fra-1 interacting proteins are identified by mass spectrometry.

4 RESULTS AND DISSCUSSION

4.1 PAPER I: C-JUN/AP-1 OVEREXPRESSION REPROGRAMS ERα SIGNALING RELATED TO TAMOXIFEN RESPONSE IN ERα-POSITIVE BREAST CANCER

Estrogen receptor has been shown to modulate gene expression through a tethering mechanism, in which ER interacts with other transcription factors (e.g. AP-1) and then binds to non-ERE sequence. However, the genome-wide overlap in DNA binding profiles of ERα and AP-1 transcription factors has not been reported.

To be able to identify how ERα signaling interplays with AP-1 signaling at the molecular level in ERα positive breast cancer cell lines, we generated an MCF-7 cell line with Tet-off-induced c-Jun protein expression. The expression and intracellular localization of c-Jun and ERα were confirmed by the Western blot analysis. The results of WST-1 cell proliferation assays performed in c-Jun-overexpressing (MCF-7/c-Jun -Tet) cells were consistent with the known effects of c-Jun in ERα-positive cells to increase cell proliferation. Furthermore, we found that the overexpressed c-Jun resulted in reduced sensitivity of MCF7 cells to tamoxifen by performing clonogenic cell survival assays.

To further analyze the cistromes of ERα and c-Jun, ChIP-seq was performed in the presence of vehicle, E2 or tamoxifen. 78% (24742 sites) of the E2-ERα bindings were identified as the tamoxifen-ERα binding regions. This is consistent with the previous study showing that the cistrome of E2 bound ERα is highly overlapped with that of tamoxifen bound ERα [334]. Similarity, 79% (19219 sites) of c-Jun binding regions under E2 treatment overlapped with that in presence of tamoxifen. Comparing the cistrome data of ERα and c-Jun treated with E2 revealed that 51% (12394 sites) of the c-Jun cistrome overlaps with the ERα cistrome. On the other hand, either ERα or c-Jun had its unique cistrome comprised of 19391 and 11913 binding sites, respectively. Motif enrichment analysis showed that ERα/c-Jun shared binding sites were more enriched in binding motifs for AP-1 (34%) than for EREs (7%) supporting the importance of indirect ERα recruitment via tethering to AP-1. Interestingly, the FOXA1 motif was enriched in shared and unique binding sites of ERα or c-Jun. In addition, GATA3 motif was enriched in ERα/c-Jun shared and ERα unique binding sites but not in unique c-Jun binding sites.

Furthermore, we found that c-Jun overexpression reprogramed ERα chromatin binding and modulated ERα-mediated gene regulation. We compared the ERα cistrome in c-Jun overexpressing cells (31785 binding sites) with ERα cistrome in parental MCF7 cells (18236 binding sites) reported in our previous study [335], which indicated that an overall increase in number of ERα binding sites and a robust redistribution of ERα binding to novel genomic loci upon c-Jun overexpression. Motif analysis of common binding sites shared by the novel gained ERα and c-Jun revealed the enrichment of AP-1 and FOXA1 motifs rather than ERE motif. These results confirmed that estrogen/ERα-dependent signaling crosstalk with AP-1 is mediated through the tethering of ERα to DNA bound

AP-1. Moreover, the mechanisms of reprogramming ERα cistrome might be due to that AP-1 motif-containing regions are opened up by c-Jun, which can permit the interaction between ERα and c-Jun or ERα directly interact with EREs located within these regions. Thus, ERα availability for other genomic regions is reduced. In addition, we analyzed E2-regulated transcriptome data (E2 vs. vehicle) in MCF-7/c-Jun -/+Tet and found that overexpression of c-Jun promoted de novo E2 regulation of genes, 290 E2-upregulated genes and 317 E2-downregulated genes only in cells with c-Jun-overexpression. Functional annotation of the 290 genes indicated that these genes are associated with cell division and mitotic nuclear division, which was consistent with our phenotypic studies.

Except the role of overexpressed c-Jun in promoting tumor cell growth, the phenotypic studies also showed that c-Jun overexpression contributed to tamoxifen resistance. Therefore, we analyzed the c-Jun-induced transcriptomic profile and found that the 266 upregulated genes significantly overlapped with the gene set upregulated in tamoxifen-resistant MCF7 xenograft tumors [221]. Moreover, integrating data from cistrome, transcriptome and clinical data revealed that TGFBI is a target gene regulated by c-Jun-reprogrammed ERα binding events associated with tamoxifen resistance. RT-qPCR and ChIP-qPCR results verified that TGFBI mRNA levels were increased upon c-Jun overexpression and both ERα and c-Jun bound to TGFBI gene. We further found that, in c-Jun-overexpressing breast cancer cells, the sensitivity of cells to tamoxifen is enhanced by knockdown of TGFBI. By performing immunofluorescence staining using tissue microarray, we also showed that the TGFBI expression level is higher in breast cancer than that in normal breast tissues. Similar finding has also been reported in other studies. For instance, it has been shown that TGFBI knockdown is associated with paclitacel resistance, a chemotherapeutic agent, in SKOC-3 ovarian cancer cells [251]. Some studies have shown that TGFβ pathway is related with chemotherapy resistance [336, 337].

In summary, a novel genome-wide footprint of ERα and AP-1 crosstalk is provided here and our data further suggest AP-1 and TGFBI signaling as investigational points of treatments in AP-1-overexpressing ERα-positive breast cancer.

4.2 PAPER II: ENDOGENOUS INTERACTION PROFILING IDENTIFIES DDX5 AS AN ONCOGENIC COACTIVATOR OF TRANSCRIPTION FACTOR FRA-1

Our previous study has shown that Fra-1 and c-Jun are highly expressed in TNBC [214], and AP-1 also plays a pivotal role in tumorigenesis. Understanding the interactions of protein-protein are fundamental to study the mechanism of AP-1 regulated gene expression. In this study, for the first time, we reported the interactome of Fra-1 in TNBC cells, which is important to detect novel coregulators of Fra-1.

Firstly, we performed RIME experiments to explore chromatin-bound partners of Fra-1 in BT549 cells and identified 118 Fra-1 associated proteins significantly enriched compared with IgG IP control. These proteins are divided in 13 groups based on PANTHER protein class analysis. For example, 50 proteins are nucleic acid-binding proteins and 13 proteins

are transcription factors. These proteins were clustered to several groups according to biological functions, such as DNA repair proteins, mRNA processing/slicing proteins and proteins related with transcription regulations. These results are important to decipher molecular mechanisms of Fra-1 signaling in TNBC cells, as the composition of Fra-1 protein complex can influence the Fra-1 regulation of downstream targets.

Our study further showed that DDX5, belonging to the DEAD-box protein family of RNA helicases, was the most enriched Fra-1 interacting protein. Furthermore, we performed ChIP-seq for Fra-1 or DDX5 in BT549 cells, respectively, which is the first genome-wide map of Fra-1- and DDX5-binding sites in TNBC cells. Comparing the cistromes of Fra-1 (30571 sites) and DDX5 (10836 sites) found that 62% of DDX5 binding sites overlapped with that of Fra-1. Genomic distribution analysis showed that Fra-1/DDX5-shared binding sites were more enriched at active promoter/5' UTR regions compared with unique binding sites of Fra-1 or DDX5. Furthermore, motif enrichment analysis revealed that the most significantly enriched motif was consensus AP-1 motif in unique Fra-1 binding sites and Fra-1/DDX5-shared-binding sites, in agreement with direct DNA binding of AP-1 protein to these regions. Regarding the unique DDX5-binding sites, CTCF motif was the most significant one, which is consistent with previous finding that DDX5 can form a complex with CTCF [338]. Above results provide evidences for a mechanism of DDX5 recruitment to DNA through interaction with Fra-1.

Moreover, based on the transcriptome data, we identified that DDX5 shares a substantial set of Fra-1 target genes and DDX5 in general has the same direction effects as Fra-1 on regulation of shared genes. These co-regulated genes are highly associated with TNBC cell growth. We already knew that deletion of Fra-1 could suppress the TNBC cell proliferation [214]. Therefore, to detect the DDX5 effects on Fra-1-driven cell growth, we performed WST-1 cell proliferation assay and clonogenic cell survival assay with specific or combined knockdown (KD) of Fra-1 or DDX5. The results demonstrated that DDX5 promotes cell growth and enhances the effect of Fra-1 on cell growth.

Furthermore, by performing AP-1 reporter luciferase assay, we found that AP-1-dependent transcriptional activation was enhanced when DDX5 was overexpressed, and knockdown of DDX5 reduced the activation. DDX5 had coactivating effect on endogenous AP-1 regulated genes containing shared Fra-1/DDX5 binding sites. In addition, the ChIP-qPCR results showed that Fra-1 KD decreased the recruitment of DDX5 to the co-occupied regions in co-regulated genes. However, DDX5 KD did not influence Fra-1 enrichment at the same regions. Previous studies have shown that DDX5 can act as a transcriptional coactivator for several transcription factors, such as AR, ER and P53, etc [299, 301]. Totally, above evidences support that DDX5 acts as a coactivator of Fra-1, and Fra-1 is essential for DDX5 recruitment to the common genomic targets.

The further integration of cistrome and transcriptome data showed that DDX5 direct target genes interacted with chromatin, including 220 downregulated and 260 upregulated genes by DDX5 KD containing DDX5 binding sites. Gene ontology analysis suggested that

DDX5 direct target genes are involved in functions related with tumor characteristics, including “regulation of cell migration”, “regulation of cell proliferation” and “regulation of cell adhesion”. Additionally, the results of immunoprecipitation staining showed the expression level of DDX5 protein is upregulated in basal like-type breast cancer, compared with non-basal like tumors. Clinically, DDX5-regulated gene expression set in breast cancer predicts clinical outcomes. Therefore, high expression of DDX5 is related with the more aggressive subtypes. It is highly likely that DDX5 promotes Fra-1-dependent breast cancer cell growth through enhancing the transcriptional activity of AP-1.

Together, we elucidated the first interactome data of Fra-1 in TNBC cell. DDX5 acts as a transcriptional coregulator of AP-1-mediated gene expression and promotes cell growth of TNBC. Thus, these findings suggest that strategies to interfere with this function of DDX5 may be of therapeutic benefit.

4.3 PAPER III: ERα AND ERβ EXERT DIFFERENTIAL REGULATION OF GENE EXPRESSION IN MCF7 CELLS

In previous *in vitro* studies, the most often used breast cancer cell models are co-expressing endogenous ERα and recombinant ERβ, which is not sufficient to provide comprehensive information on the specific roles of ERβ homodimers in ERα-positive cells. Therefore, in this study, we generated a novel breast cancer cell model, the only ERβ-expressing MCF7 cell line, to further complement the understanding of the unique functions of ERα or ERβ in ERα-positive breast cancer cells.

First, to generate the only ERβ-expressing MCF7 cell line, we used CRISPR/Cas9 system to knock out ERα in the Tet-Off-inducible ERβ MCF7 cell line. The reasons why we choose the Tet-Off-inducible ERβ MCF7 cell line are as follows, first, the cell line was generated and available in our lab [339]; second, this cell line contains Tet-off system, which can express endogenous ERα and inducible ERβ in the absence of Tet. DNA sequencing showed that the transfected sgRNA that targeted on exon 1 of *ESR1* gene caused frameshifting insertions to stop the expression of ERα. In line with this, the cell model was also validated by the Western blot analysis. Therefore, in the absence of Tet, the cell model only expresses ERβ without ERα expression.

Furthermore, we selected four known estrogen-responsive genes, including *PKIB*, *PS2*, *IL20* and *GREB1*, to examine the role of ERβ in regulation of gene expression. E2 stimulation upregulated the gene expression of *PS2*, *GREB1* and *PKIB* while downregulated *IL20* expression mediated through ERβ. This confirmed that ERβ was functional to regulate these genes upon the E2 stimulation in the absence of ERα, which supports that the transactivation of ERβ homodimer is ligand-dependent.

Since the role of ERβ on cell proliferation is still inconsistent to date, we performed WST-1 assay to assess the proliferation phenotype in only ERα- or ERβ-expressing MCF7 cells. We found that only ERβ expression reduced the cell growth rate compared with only ERα-expressing MCF7 cells cultured in full-serum medium. In addition, we found that only

ERβ-expressing MCF7 cells displayed a significant reduction in cell proliferation in response to E2 compared with vehicle. Conversely, only ERα-expressing MCF7 cells displayed an increased cell proliferation upon E2 treatment. Therefore, in our study, ERα and ERβ have opposing roles on E2-dependent cell proliferation.

Moreover, we performed RNA-Seq analysis in hormone deprived only ERα- or ERβ-expressing MCF7 cells with E2 stimulation. The data revealed that 1359 and 1567 genes was regulated by only ERα or ERβ in response to E2 ($|FC|>2$, $FDR<0.05$), respectively. We also found that 31% of E2-upregulated genes in only ERβ-expressing MCF7 cells overlapped with that in only ERα-expressing MCF7 cells, and 45% of E2-downregulated genes modulated by ERβ overlapped with that in only ERα. These results indicated that ERβ can modulate specific gene expression profile different from that of ERα. In addition, the results of functional enrichment analysis for two ER isoform-specific E2-upregulated genes showed that either ERα or ERβ uniquely modulated genes have various molecular and cellular functions. The results of GO analysis further indicated that two ER isoforms function in opposite direction to regulate cell proliferation. The ERα-specifically upregulated genes are enriched in the biological process "positive regulation of cell proliferation", consistent with the known effect of E2 in ERα-positive cells. While the gene set that specifically upregulated by ERβ are significantly involved in the biological process "negative regulation of cell proliferation", in line with the observed phenotypic differences.

In conclusion, the significance of this study is the generation of novel only ERβ-expressing MCF7 cell model. This cell model is foundational resource for us to detect the specific roles of ERα and ERβ in ERα-positive breast cancer cells. Our study also suggests that two ERs isoforms have opposite effects on cell proliferation through regulating distinct sets of target genes in response to E2.

5 CONCLUDING REMARKS AND PERSPRECTIVES

5.1 AP-1 IN ERα-POSITIVE BREAST CANCER

A critical mechanism of ER signaling, tethering mechanism, has been proposed, in which ligand-activated ERα indirectly interacts with DNA via other transcription factors, such as AP-1. In this thesis, we investigated the genome-wide assessment of c-Jun, a potent member of AP-1 family, and ERα cistrome and transcriptome in ERα-positive breast cancer cells, which demonstrate the co-localization of ERα and c-Jun binding regions at genome-wide level and suggests that ERα tethering to AP-1 is a global mechanism for gene transcription regulated by ERα. In addition, the results showed that the sensitivity of ERα-positive breast cancer cells to tamoxifen therapy is reduced by the overexpression of c-Jun. This is consistent with earlier findings that enhanced AP-1 transcriptional activity is associated with endocrine therapy resistance [209, 221]. Moreover, it is shown that TGFBI is associated with poor outcomes of ERα-positive breast cancer patients receiving endocrine therapy and a potential gene that may cause tamoxifen resistance through the crosstalk of ERα and AP-1.

In summary, this thesis highlights a role of AP-1 and ERα crosstalk through regulation of TGFBI expression as a potential contributor of endocrine resistance in ERα-positive breast tumors. On the other hand, it has previously been indicated that TGFBI can function as a chemo-sensitizer or a risk of chemo-resistance in various cancers [227, 228]. Therefore, a more comprehensive understanding of TGFBI signaling mechanism will be essential to identify novel strategies to overcome endocrine therapy resistance.

5.2 AP-1 IN TNBC

Fra-1, a member of AP-1 transcription factors, has been shown to be overexpressed in TNBC and associated with poor prognosis [214]. The downstream gene regulation of Fra-1 can be influenced by associated coregulators and thus the alterations in the composition of Fra-1 protein complex may have direct clinical implications. In this thesis, the first interactome data of Fra-1 in TNBC cells is reported, highlighting that DDX5 is the most enriched interacting protein of Fra-1. Furthermore, it is shown that DDX5 has a role of transcriptional coactivator for Fra-1, which is able to enhance Fra-1-dependent TNBC cell proliferation through increasing the transcriptional activity of Fra-1.

As we know, currently, there is still no effective target therapy for TNBC. This thesis also shows that DDX5 expression level is higher expressing in triple-negative basal-like tumors than that in non-basal-like ones. In addition, the direct target gene set of DDX5 can predict poor clinical outcome of breast cancer patients. Moreover, interfering with the DDX5 coregulatory effect for Fra-1 could alter AP-1 mediated gene expression involved in tumor progression. Therefore, DDX5 might be a candidate for targeting therapy in TNBC patients.

5.3 ERβ IN ERα-POSITIVE BREAST CANCER

In breast cancer, the function of ERα is more clearly defined compared with ERβ. In this thesis, we generated a novel cell model, only ERβ-expressing MCF7 cell line, using CRISPR/Cas9 system to knock out ERα. This cell model is better to study the functions of ERβ homodimers in ERα-positive breast cancer cells, compared with most previously used cell models which contain co-expression of ERα and ERβ. Therefore, this novel cell model is an important resource to further complement the understanding of the roles of two different ER isoforms in ERα-positive breast cancer cells. It also provides the evidence that two ERs have opposite effects on E2-dependent cell proliferation by regulating distinct sets of target genes in respond to E2. The different pattern of gene expression profile uniquely regulated by ERα or ERβ is fundamental. It is essential to further study the mechanism of two different ER isoform signaling, especially the target genes and signaling pathway associated with tumor cell growth, to identify the novel strategies for breast cancer therapy.

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